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Research article

Macrophages exposed continuously to lipopolysaccharide and other agonists that act via toll-like receptors exhibit a sustained and additive activation state

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Abstract

Background: Macrophages sense microorganisms through activation of members of the Toll-like receptor family, which initiate signals linked to transcription of many inflammation associated genes. In this paper we examine whether the signal from Toll-like receptors [TLRs] is sustained for as long as the ligand is present, and whether responses to different TLR agonists are additive.

Results: RAW264 macrophage cells were doubly-transfected with reporter genes in which the IL-12p40, ELAM or IL-6 promoter controls firefly luciferase, and the human IL-1 β promoter drives renilla luciferase. The resultant stable lines provide robust assays of macrophage activation by TLR stimuli including LPS [TLR4], lipopeptide [TLR2], and bacterial DNA [TLR9], with each promoter demonstrating its own intrinsic characteristics. With each of the promoters, luciferase activity was induced over an 8 hr period, and thereafter reached a new steady state. Elevated expression required the continued presence of agonist. Sustained responses to different classes of agonist were perfectly additive. This pattern was confirmed by measuring inducible cytokine production in the same cells. While homodimerization of TLR4 mediates responses to LPS, TLR2 appears to require heterodimerization with another receptor such as TLR6. Transient expression of constitutively active forms of TLR4 or TLR2 plus TLR6 stimulated IL-12 promoter activity. The effect of LPS, a TLR4 agonist, was additive with that of TLR2/6 but not TLR4, whilst that of lipopeptide, a TLR2 agonist, was additive with TLR4 but not TLR2/6. Actions of bacterial DNA were additive with either TLR4 or TLR2/6.

Conclusions: These findings indicate that maximal activation by any one TLR pathway does not preclude further activation by another, suggesting that common downstream regulatory components are not limiting. Upon exposure to a TLR agonist, macrophages enter a state of sustained activation in which they continuously sense the presence of a microbial challenge.

Background

Mammalian macrophages respond to a wide range of mi-

crobial products with induction of genes required for host defence. Amongst these genes is a suite of inducible

cytokines, including IL-1, TNF- α , IL-6 and IL-12, which are required for protective innate and acquired immunity but also mediate much of the pathology of disseminated infections including toxic shock [1]. The archetypal macrophage activating bacterial product is lipopolysaccharide (LPS) or endotoxin. An understanding of the mechanism of action of LPS was greatly expedited by the identification of the Toll-like receptor [TLR] family and the parallels between recognition of bacterial stimuli in mammals and drosophila. Mice with mutations in the TLR4 gene are hypo-sensitive to LPS [2–4]. Activation of TLR4 on the cell surface which requires cooperation with at least two other surface proteins, CD14 and MD2 [5] leads to recruitment of adaptor proteins (MyD88, IRAK, TRAF6) that ultimately couple the recognition of the microbial product to activation of the transcription factor complex NF- κ B [Reviewed in [6]]. Translocation of this transcription factor complex in turn contributes to activation of the promoters of many inducible genes in macrophages [1]. Another member of the TLR family, TLR2, is absolutely required for recognition of a range of surface components of gram-positive organisms including bacterial lipopeptides [7]. Apart from bacterial cell wall components, macrophages are able to recognize and respond to bacterial DNA or CpG-containing immunostimulatory oligonucleotides [8,9]. A recent paper indicates that yet another member of Toll-like receptor family, TLR9, is required for optimal recognition of immunostimulatory DNA [10]. The TLR2, TLR4 and TLR9 pathways apparently converge at the level of MyD88, since macrophages from mice with a mutation in this gene are defective in activation by either gram-negative or gram-positive organisms or bacterial DNA [11,12].

One clinically-important feature of the mammalian response to LPS is the induction of tolerance. An animal exposed to a sub-lethal dose of LPS, or to a non-toxic LPS, enters a refractory period during which it can resist lethal LPS exposure. Several groups have attempted to model the phenomenon of endotoxin tolerance *in vitro* by studying the ability of macrophages exposed to LPS to recognise a secondary stimulus [5,13–22]. In these models, some responses that are measured (e.g. activation of transcription factor NF- κ B, TNF- α mRNA production) occur transiently after LPS addition. Whereas TNF- α mRNA is induced transiently in response to LPS and then rapidly degraded [23] and the protein product is produced and secreted in a bolus from the stimulated cells [24], other mRNAs are elevated for as long as LPS is present, up to 48 hrs after LPS addition [23]. Such genes could be induced by autocrine stimuli produced in the initial phase of activation. Alternatively, LPS may act to cause a change of "steady state", and TNF- α induction and other early response genes could be a feature of a transition state that cannot be reactivated until the fully

active state is allowed to completely decay. In such a model, the new steady state could have an intrinsic half-life or it could be maintained by continued stimulation. Explanations for tolerance in macrophages *in vitro* generally involve selective modulation or repression of some component of the signalling cascade, ranging from the putative LPS receptor TLR4, through IRAK and various regulators of NF- κ B [[5,17] and references therein]. Many of the proposed mechanisms of tolerance would imply cross-desensitisation of responses to other bacterial products presumed to share the same pathway, a phenomenon that has been reported between TLR2 and TLR4 [25,26].

It is self-evident that if genes such as TNF- α and *c-fos* are induced transiently by LPS, there is a state that might be called "tolerance" or "repression" in that the mRNA and protein declines despite the continued presence of the stimulus. The transitory nature of TNF- α induction is probably a consequence of induction of nucleases that specifically degrade the mRNA [27]. Such a feedback mechanism would clearly interfere with subsequent induction by any stimulus for as long as the nuclease activity was retained. By contrast, where mRNAs continue to increase and there is no evidence of feedback control, there is no reason to expect any restriction on additive signaling by different TLR agonists unless there is an intrinsic limit to the amount of mRNA that can be made or common pathway components are limiting. There is no obvious reason why it would be desirable for macrophages to restrict their ability to recognise more than one TLR-related challenge simultaneously, especially in mixed infections. Based upon these considerations, we hypothesised that genes that are induced in a sustained manner by TLR agonists would not display any kind of "tolerance" and that there would be significant advantage to macrophages in being able to integrate multiple signals that induce such genes.

To address this hypothesis, we have developed novel reporter gene systems. The firefly luciferase gene product is very unstable in the macrophage cell line RAW264, and stably-transfected cells have provided a sensitive indicator of transient activation of κ B-dependent transcription in these cells [28]. We examined the regulation of promoters of the IL-1 β , IL-6 and IL-12p40 genes as well as the widely-used κ B-responsive gene, ELAM (E-selectin) in stably-transfected RAW264 cells. We demonstrate that sustained activation of the promoters of these genes requires the continued presence of microbial agonist and that signaling by one such agonist does not preclude additional activation through a distinct Toll-like receptor pathway. In the second part of the study, we use constitutively active forms of TLRs to generate the primary activation of reporter gene expression, and

again show that their actions are additive with those of agents that act through other TLRs. Finally, we show that primary macrophages can, indeed, respond to multiple TLR stimuli with increased cytokine secretion. In overview, we conclude that macrophages sense microbial challenges continuously and can respond to more than one stimulus simultaneously.

Results

Generation and characterisation of stably transfected RAW264 cell lines with two integrated luciferase reporters

Previous studies examined a stable transfectant of the cell line RAW264, in which the firefly luciferase gene directed by the NF- κ B-dependent HIV-1-LTR was integrated into the genome. This line provided a sensitive indicator of response to LPS and to CpG DNA [8,28]. Induction of luciferase activity was transient, reaching a peak after around 2 hrs and then declining rapidly to control levels. The time course of luciferase activation was consistent with transient induction of nuclear NF- κ B activity [8,28] and TNF- α mRNA [8,28] demonstrated previously using RAW264 cells cultured in similar conditions. Although NF- κ B is strongly implicated in regulated gene expression in macrophages, numerous other transcription factors (i.e. PU.1, Ets-2, Sp1, Stat-1, C/EBP β , γ or δ , IRF-1 etc [1,29]) are regulated or induced in LPS-stimulated cells, so we decided to examine more complex promoters that are not solely dependent upon NF- κ B.

To examine genes that are induced at a transcriptional level in RAW264 cells, we made a series of stable RAW264 cell transfectants with cytokine promoters driving luciferase. Stable transfection avoids the complication that derives from the ability of macrophages to recognise and respond to plasmid DNA [8]. The interleukin-1 β gene is of particular interest, because previous reports indicate that "LPS tolerance" does not prevent re-induction of this gene or of the interleukin 6 gene [30,31]. We cloned the human interleukin-1 β promoter into a renilla luciferase plasmid, which allowed us to produce lines in which the IL-1 β induction response could be measured simultaneously with other promoters that may, or may not, exhibit tolerance.

RAW264 cells were cotransfected by electroporation with two separate reporters and after 2 weeks of selection in G418, several hundred foci of stable transfectants were pooled for further study. Because of the known variation between RAW264 subclones in terms of LPS-inducible gene expression [23] we chose not to study single cell clones. In a separate study, we have confirmed that all clones derived from the pools used in this study express both the firefly and renilla luciferase genes at low levels, but vary considerably in whether that activity is

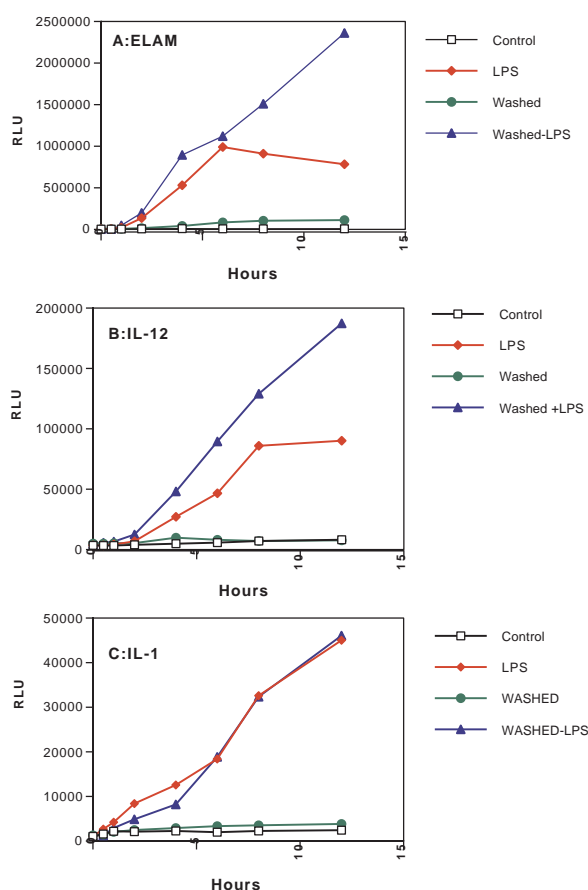
inducible by LPS or other agonists [32]. The expression of luciferase and responsiveness to microbial challenge has been stable in the pools of transfectants for at least 2 months in continuous culture. To our knowledge, this is the first example of the use of multiple reporter genes in a stably-transfected macrophage line.

Time and dose response curves for activation of luciferase expression

Comparative time and dose response curve analysis were performed for each of the pooled transfectant lines with different combinations of promoters. Each promoter controlling firefly luciferase behaved in a unique manner, regardless of the presence of the IL-1 promoter-renilla luciferase gene in all of the lines, whereas the IL-1 promoter regulation was remarkably consistent in independent lines with different combinations of firefly luciferase reporters.

The ELAM-1 promoter, used commonly as an indicator of κ B-dependent transcription [33], displayed the highest basal activity and inducibility. In our studies of the HIV-1-LTR, we found that adherence to tissue culture plastic was stimulatory [unpublished], so the transfected RAW264 cells were plated in the evening and stimulated the next morning after the cells were fully adherent. We considered the possibility that overnight culture could lead to accumulation of endogenous stimulatory or inhibitory cytokines, so we examined the effect of replacing the medium. The outcome depended upon the reporter gene. When cells were exposed to fresh medium, ELAM-luciferase activity decreased transiently, then increased continuously over a 12 hr incubation (this is not obvious in Fig 1A, because of the much larger effect of added LPS). Following addition of 100 ng/ml LPS to unwashed cells, ELAM-luciferase was detectably elevated 2-fold after 30 mins, and continued to increase relative to unstimulated expression for up to 6–8 hrs, after which it declined (Fig 1A). In cells provided with fresh medium, the tail-off at 6–8 hrs did not occur, and luciferase activity was still increasing at 12 hrs. Hence, endogenous regulators appear to constrain activation of the NF- κ B-dependent promoter. In the same stably-transfected cells, the IL-1 β renilla luciferase did not show either the rapid decline upon medium replacement, or the increase in basal activity thereafter (Fig 1C). However, fresh medium did accelerate the response to LPS, and permit the response to continue rising up to 12 hrs.

Fig 1B shows a comparable time course for the IL-12 promoter. The data for the IL-1 β promoter in this pool of stable transfectants line were indistinguishable from those obtained with the ELAM/IL-1 transfectants, and have been averaged in Fig 1C. Unlike the IL-1 promoter in the same cells, the response of the IL-12 promoter to LPS

**Figure 1**

Time course of activation of integrated reporter genes in RAW264 cells. Pooled stable transfectants of RAW264 cells, with either the ELAM or IL-12 promoter driving firefly luciferase, cotransfected with the IL-1 β promoter driving renilla luciferase, were cultured overnight as described in Materials and Methods. Where the cells were washed, the medium was aspirated, and replaced immediately with warm medium at time zero. A typical experiment of three is shown. Results for ELAM and IL12 promoters are the average of duplicates that differ by less than 10% from the mean. In the case of the IL-1 β data, the results are the average of 4 datapoints obtained with the two separate pooled transfectant lines in the same experiment.

was also increased marginally by washing and continued to increase when the control activation had peaked. Similar results were obtained with a population of cells transfected with an IL-6 firefly luciferase reporter together with the IL-1 renilla luciferase reporter (data not shown). These findings suggest that the RAW264 cells produce a feedback suppressor of LPS response that has some selectivity for the target promoter being studied. Because the introduction of fresh medium, or use of freshly plated-cells creates a rising baseline which is dif-

ficult to interpret, we chose a standard assay procedure in which the cells were plated late in the afternoon and stimulated the next day for 8 hrs without changing the medium. The sensitivity of the assay allowed us to obviate the effect of accumulated inhibitors following overnight culture via the use of relatively low starting cell density (2×10^5 /ml).

Reporter gene lines respond to a wide range of microbial agonists with distinct dose response curves

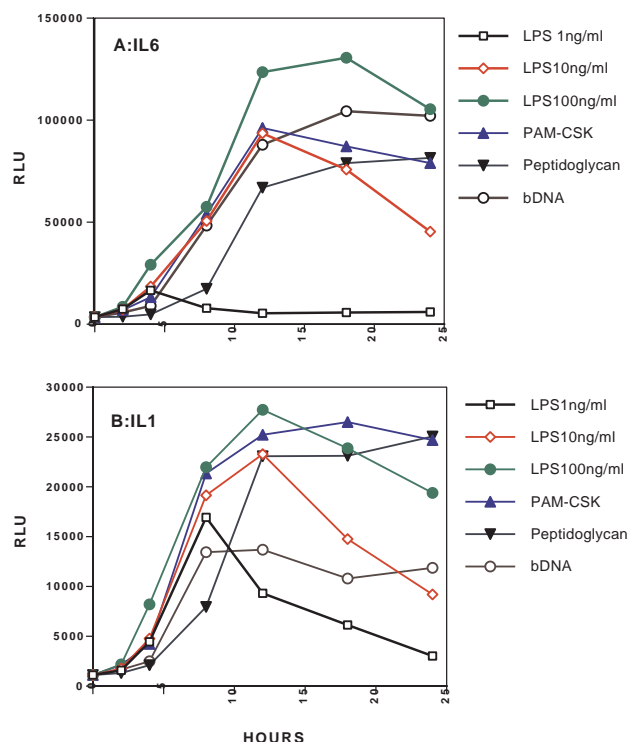
The different indicator cells lines each responded to a wide diversity of different agonists of bacterial origin in addition to LPS, including the synthetic bacterial lipopeptide, PAM₃-CSK₄ [34], peptidoglycan and bacterial DNA (bDNA) or CpG-containing oligonucleotides. Figure 2 shows an example using the IL-6 promoter in combination with the IL-1 β promoter in the same cells. This study makes several additional points:

- 1) Activation of expression of both reporter genes was detectable in response to LPS at 1 ng/ml.
- 2) The response of both promoters to low doses (1 or 10 ng/ml) of LPS was transient and declined after 10–15 hrs, where the response to higher LPS concentration (100 ng/ml) was sustained for longer and was still almost maximal after 24 hrs. Hence, the duration, rather than the peak magnitude, of the response was most sensitive to LPS concentration.
- 3) The different agonists have different relative activities on the two promoters. In keeping with the previous observation that IL-1 β is weakly induced by bDNA [8], bDNA was as effective as LPS at inducing IL-6 promoter but only half as effective on the IL-1 promoter.

Figure 3 shows dose response curves for LPS, lipopeptide and bacterial DNA for the IL-12, IL-1 and ELAM-1 promoters. Activity of both the IL-1 and IL-12 promoters continued to increase up to 500 ng/ml LPS whereas the activation of ELAM luciferase was detectable at 0.1 ng/ml and maximally at 10 ng/ml. Given that the ELAM-1 promoter is apparently more sensitive to LPS than other promoters tested, the rising baseline seen upon addition of fresh medium (Fig 1) is likely to be due to endotoxin and/or endotoxin-like activity in serum, which cannot be completely avoided and which the cells themselves degrade with time.

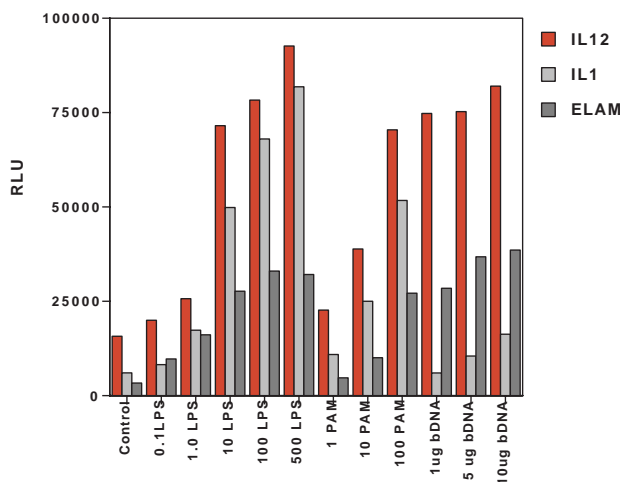
The effects of pretreatment

The main purpose of creating these lines of RAW264 cells is to use them as convenient indicators of interactions amongst signals generated by different microbe-associated stimuli. We focused upon the IL-12/IL-1 and ELAM-IL-1 lines, which appear to display the spectrum

**Figure 2**

Time course of activation of IL-6 and IL-1 promoters in RAW264 cells and the effect of a range of microbial agonists. Pooled stable transfectants with the IL-6 promoter driving firefly luciferase and the IL-6 promoter driving renilla luciferase were incubated overnight then stimulated with the agents shown for the time indicated prior to assay of luciferase activity. PMA3-CSK was added at 100 ng/ml, peptidoglycan at 10 µg/ml and E-coli genomic DNA (bDNA) at 10 µg/ml. A representative experiment of two is shown. Datapoints are the average of duplicate wells.

of contrasting activation patterns. Each line was incubated overnight (16–18 hrs) with 100 ng/ml LPS, 100 ng/ml lipopeptide or 10 µg/ml of bacterial DNA, washed twice with fresh medium, and then re-exposed to a range of agonists. The results are shown in Fig 4. Even after 18 hrs, luciferase activity was still elevated at least 10-fold in all cases. In cells washed to remove LPS, the elevated activity declined by about 70% after 8 hrs, but immediate re-addition of LPS maintained activity of the ELAM-1, IL-12 and IL-1 promoters. By contrast to the pattern observed in LPS-stimulated cells deprived of stimulus, ELAM, IL-12 or IL-1β promoter luciferase activity stimulated by lipopeptide did not decline greatly after removal of the stimulus. bDNA behaved intermediately for all promoters; activity declined 20–60% after removal of the stimulus.

**Figure 3**

Comparative dose response curves for activation of IL-12, IL-1 and ELAM promoters. Pooled stable transfectants of RAW264 cells, with either the ELAM or IL-12 promoter driving firefly luciferase, cotransfected with the IL-1β promoter driving renilla luciferase, were cultured overnight as described in Materials and Methods. E. coli genomic DNA (bDNA), LPS or PAM3-CSK lipopeptide (PAM) were added at the concentrations indicated (LPS and PAM are in ng/ml, bDNA in µg/ml). After 8 hrs, the cells were harvested for determination of luciferase activity. Datapoints are the average of duplicate wells. The experiment is representative of two, and reiterates the pattern observed in 2 others with differences in design.

Addition of a different agonist revealed a surprisingly simple pattern of purely additive responses. This is most obvious in the case of lipopeptide-treated cells, where maximal activity was retained despite removal of the primary agonist. Addition of either LPS or bDNA caused an almost additive increase in luciferase activity, whereas addition of peptidoglycan (which probably shares the TLR2-signalling pathway with lipopeptide) was without additional effect (data not shown).

Interaction of different agonists with the Toll-like receptor pathways

In a separate report, we have examined the ability of dominant-positive forms of the Toll-like receptors [TLRs] to mimic actions of the microbial agonists in macrophages [35]. Activation was achieved by fusing the extracellular domain of CD4, which promotes spontaneous dimerisation, with the intracellular domain of TLRs. CD4-TLR4 alone was able to activate production of the cytokine TNF-α, and ELAM luciferase activity. By contrast, CD4-TLR2 was active only when transfected in

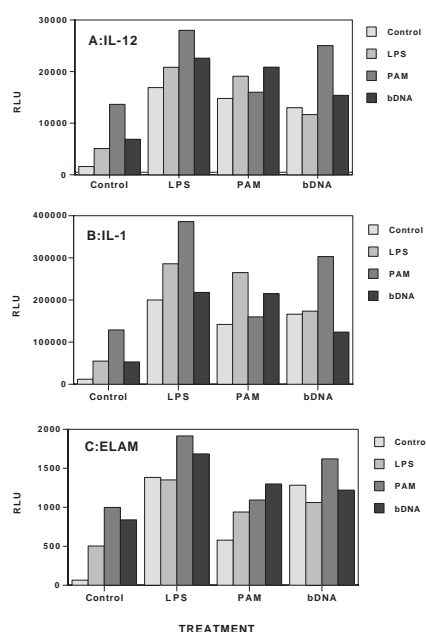


Figure 4

Additive effects of different microbial agonists and lack of evidence of tolerance in reporter gene activation. Pooled stable transfectants of RAW264 cells, with either the ELAM or IL-12 promoter driving firefly luciferase, cotransfected with the IL-1 β promoter driving renilla luciferase, were cultured overnight for 16–18 hrs with either no addition, LPS (100 ng/ml), PAM3-CSK lipopeptide (PAM, 100 ng/ml) or *E. coli* genomic DNA (bDNA, 10 μ g/ml) as indicated on the vertical legend. The cells were washed by aspirating the medium, replacing it with warm medium, incubating for 5 minutes and replacing the medium a second time. The replacement medium contained no additional stimulus, or one of original stimuli as indicated on the legend to the X-axis, so that a 4 \times 4 matrix of pretreatment and retreatment was established. After a further 8 hrs stimulation, the cells were harvested for determination of luciferase activity. Each datapoint is the average of duplicate wells. The experiments is representative of two with identical design. The pattern of restimulation has been confirmed for LPS, and for the ELAM/IL-1 line in separate experiments (not shown).

combination with either TLR1 or TLR6. We investigated whether the active TLRs alone could induce IL-12 luciferase, and whether their actions were additive with their agonists or, alternatively, produced some kind of tolerance.

When the IL-12 luciferase reporter gene was co-transfected into RAW264 cells together with the the CD4-TLR4 expression vector, reporter gene expression was strongly induced (Fig 5). By contrast, CD4-TLR2 or CD4-TLR6 had little effect when added alone. When added in combination, TLR2 plus TLR6 activated IL-12 luciferase activity to the same extent as CD-TLR4 added alone. This

pattern of signaling is the same as previously described for activation of TNF- α production [35]. Because of the possible complex effect of stimulatory DNA sequences in the co-transfected plasmids, we confirmed that the response was due to expression of the protein products by inactivating each signaling domain by mutating a conserved proline in each TIR domain that corresponds to the inactivating mutation in TLR4 in the C3H/HeJ mouse strain, P712H [36,37]. We have previously shown that mutation of the conserved prolines in TLR2 and TLR6 similarly inactivates these receptors [34,35]. The ability of CD4-TLR4 to trans-activate IL-12 promoter activity was abolished by the P-H mutation. In fact, the CD4-TLR4-PH mutant expression plasmid reproducibly reduced the basal IL-12 promoter activity suggesting that the mutant receptor may generate a repressive signal. In the case of TLR2 and TLR6, co-transfection of both P-H mutant expression plasmids together also suppressed the basal activity. However, when wild-type TLR2 or 6 was co-transfected with the reciprocal P-H mutant partner, there was a small, but reproducible activation above the control; even more relative to the level observed when both partners were P-H mutants. This complex pattern strongly supports the view that the CD4-TLR2 and CD4-TLR6 heterodimerise in order to signal [35] and also suggests that the P-H mutation reduces, but does not completely abolish, the ability of either partner to contribute some function to an active heterodimer.

RAW264 cells transfected with CD4-TLR plasmids, and the IL-12 promoter, were incubated overnight, washed and treated with microbial agonists. The extent of the activation by CD4-TLR4 was comparable to that induced by addition of a maximal dose of LPS (100 ng/ml) to control cells and LPS did not stimulate further. By contrast, the effects of both lipopeptide and bacterial DNA were approximately additive with CD4-TLR4. The converse experiment gave the expected reciprocal pattern. Lipopeptide addition to cells transfected with CD-4TLR2/6 lifted the luciferase activity to the level observed when the agonist was added to control cells, whereas both LPS and bacterial DNA effects were almost perfectly additive.

Additive effects of different agonists on inducible cytokine production

Both the experiments with stable reporter gene lines, and the studies of the dominant-active TLRs, suggest that different Toll-like receptor pathways can operate independently of each other to induce inflammatory cytokines. To confirm the biological relevance of this observation, we examined the effect of combined addition of two such agonists, LPS and CpG DNA. Data in Fig 6 show that combined addition of LPS and bacterial DNA or the stimulatory CpG oligonucleotide AO-1 actually produced a more than additive release of TNF- α , IL-6 or

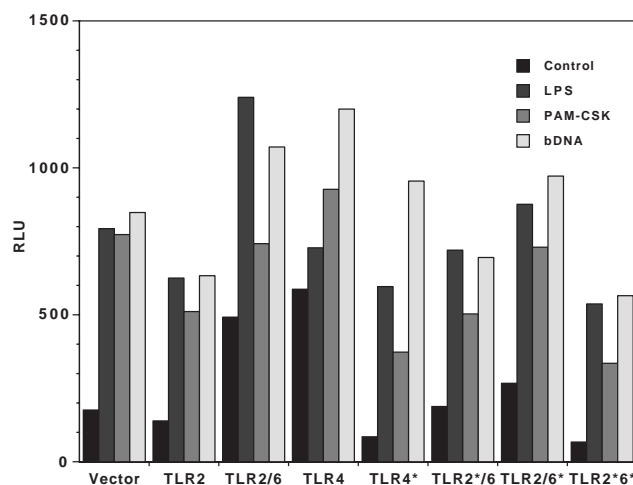


Figure 5

Combined effects of constitutively active toll-like receptors and added agonists. RAW264 cells were transiently co-transfected with the IL-12 promoter luciferase reporter genes and expression plasmids encoding wild-type and mutated (indicated by an asterisk) forms of the CD4/TLR chimaeric receptors as indicated either alone for TLR4 or in combination for TLR2 and 6. The experiment is described in detail in Materials and Methods. After overnight incubation, the cells were treated with either LPS [100 ng/ml], PAM3-CSK lipopeptide (100 ng/ml) or E-coli genomic DNA (10 µg/ml) as indicated. Each datapoint is the average of duplicate wells. The same pattern was observed in three separate experiments. The major variation between experiments was in the effect of the vector alone on basal reporter gene activity.

IL-12 protein from RAW264 cells after 24 hrs. The same pattern was evident at 8 hrs (not shown). Controls in this experiment, DNaseI-treated DNA and GpC oligonucleotide (NAO-1) confirm that the response is, indeed, due to stimulatory DNA recognition in combination with LPS. In panel D, the observation is extended to primary bone marrow-derived macrophages, to provide assurance that this is not a peculiarity of the RAW264 cell line.

Discussion

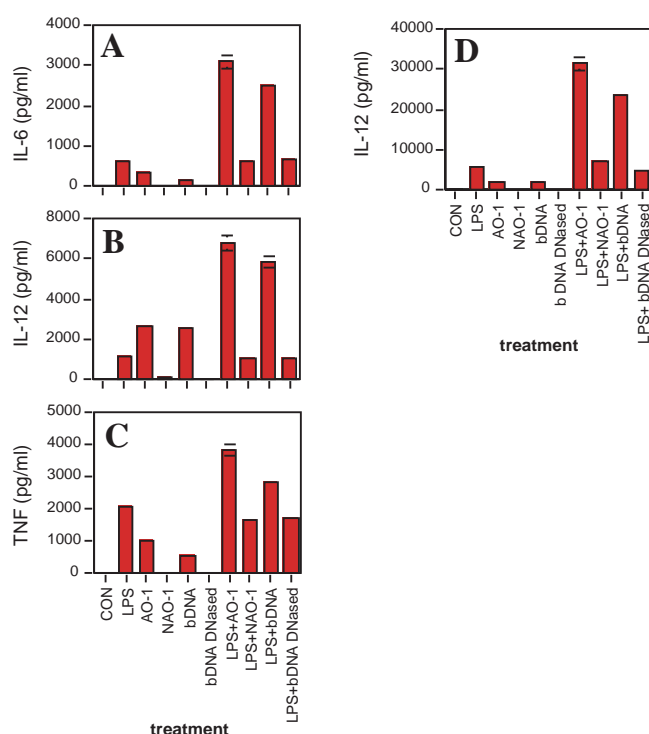
Contrasting regulation of late response genes in macrophages

Macrophages that have been treated with a maximal LPS dose *in vitro* fail to respond to restimulation in most early response assays including phosphorylation of MAP Kinases, activation of NF-κB and AP-1 transcription factors, and induction of a range of cytokine and chemokine genes [5,17]. Such responses are transient responses to LPS, and have declined to "almost" basal levels by the time of restimulation, regardless of whether LPS is

removed. By contrast, there is a family of transcription factors and target genes that are induced much more slowly by LPS, and which remain elevated at least 48 hrs after ligand addition. They include the transcription factors Ets-2, C/EBP family members and Sp1 [1,38] and the interferon-regulatory factors [29], the serpin plasminogen activator inhibitor-2 and inducible nitric oxide synthase (iNOS) [23] the cytoskeletal regulators MARCKS and MacMARCKS [39] and many of the inducible cytokines such as IL-1, IL-6, IL-10 and IL-12 and other regulators [20,21,31,40–44]. Amongst these stably-inducible genes is p50NFκB, a repressor of κB-dependent transcription, which provides at least one explanation for suppression of transcription of early response genes such as TNF-α [45].

In this study we have used a novel reporter gene system to examine how late-responsive genes are regulated. We show with a number of promoters, including the κB-dependent ELAM promoter, that the levels of luciferase activity increase for at least 8 hrs following addition of microbial agonists, and are maintained thereafter long after early events such as nuclear NF-κB activation and MAP kinase activation and induction of early response genes such as TNF-α has peaked and declined. The examples we have shown, IL-6, IL-12 and ELAM are only a subset of the promoter combinations we have tested successfully. At least for IL-1β and iNOS, the time course of luciferase activation is consistent with the time course of transcriptional activation of the corresponding gene measured previously using nuclear run-on transcription assays in the same cells [23].

The key observation in the present paper is that luciferase activity declines upon removal of the agonists LPS and bacterial DNA, and this decline can be prevented by immediate re-addition of the agonists. The observation that this is not readily apparent for the agonist PAM₃-CSK₄ may reflect something fundamentally different about its signaling pathway, or may simply reflect a reduced ability of the cell to degrade or detoxify residual amounts of this ligand. The implications of the declining signals seen after ligand removal are two-fold. Firstly the cells are not LPS-unresponsive, and secondly maintenance of the induced luciferase requires continuous stimulation by LPS (or lipopeptide or bacterial DNA as the case may be). In keeping with this interpretation, the response of the IL-1, IL-6 and IL-12 promoters to submaximal doses of LPS peaked and declined earlier (Figs 2,3) presumably because the cells consume the ligand, and the response then declines. A useful analogy can be drawn with the activation of the CSF-1 receptor in macrophages. The addition of CSF-1 to cells previously starved of ligand causes a rapid activation of signalling pathways leading to MAP kinase activation, PI-3-kinase,

**Figure 6**

Combined effects of LPS and bacterial DNA on pro-inflammatory cytokine production Panels A-C. RAW264 cells were stimulated for 24 h with medium control, LPS [100 ng/ml], stimulatory CpG oligonucleotide AO-1 (3 μ M), non-stimulatory GpC oligonucleotide NAO-1 (3 μ M), *E. coli* DNA (bDNA, 5 μ g/ml), DNase I-treated bDNA (5 μ g/ml) or combinations as indicated. Cell supernatants were harvested and IL-6 (Panel A), IL-12 (Panel B) and TNF- β (Panel C) levels were estimated by ELISA. Data are mean of triplicates \pm SD and similar results were obtained in 3 independent experiments. Panel D. Bone marrow-derived primary macrophages were stimulated with medium control, LPS (100 ng/ml), AO-1 (3 μ M), NAO-1 (3 μ M), *E. coli* DNA (bDNA, 5 μ g/ml), DNase I-treated *E. coli* DNA (5 μ g/ml), or combinations as indicated. After 24 h, cell supernatants were harvested and IL-12 levels in supernatants were estimated by ELISA. Data (mean of triplicates \pm SD) are representative of 4 independent experiments.

induction of AP-1 and Ets-2 transcription factors, and ultimately transcriptional activation of specific target genes such as the urokinase plasminogen activator gene (uPA) [46]. It is a sequential cascade, since Ets-2 must be induced before it can be phosphorylated by activated MAP kinase [46]. Ets-2 phosphorylation is also induced by LPS [38] and probably relates to the ability of LPS to prevent apoptosis in macrophages [9]. In stimulated cells, the CSF-1R binds ligand, and is rapidly internalised and degraded. The CSF-1R mRNA (encoded by the *c-fms* proto-oncogene) is substantially down-modulated (an-

other response mimicked by LPS; [9]). Yet, uPA mRNA is maintained for as long as CSF-1 is present; removal of CSF-1 leads to rapid degradation of uPA mRNA in a manner that is blocked by inhibitors of protein or RNA synthesis [47]. The implication is that after the initial signalling pulse, the cells must continue to recognise and respond to ligand in a quite distinct manner. Indeed, although the steady level of CSF-1 receptor is low, activity can be discerned from the continued degradation of the ligand by the cells [48]. In the case of LPS, recent availability of TLR4 antibody supports a similar mechanism. The initial recognition of LPS was shown to lead to rapid down-modulation of TLR4 from the cell surface and reduced expression of TLR4 mRNA [5].

Although we have questioned the nature of LPS tolerance in macrophages treated *in vitro*, the data we have obtained actually provide a plausible mechanism for LPS tolerance *in vivo*. The double reporter system enabled us to confirm and extend an earlier finding showing that early LPS responses, such as induction of TNF- α and NF- κ B-dependent transcriptional activation (e.g. part of the ELAM-1 activation and the earlier HIV-1-LTR responses) require at least 10-fold lower concentration of LPS than later responses. We suggest that early responses such as TNF- α induction are activated solely in response to an initial bolus of LPS or other agonist interacting with receptor, a response that is truly concentration dependent. By contrast, activation of late response genes, which requires the continued presence of the ligand, depends upon the amount of ligand available to each cell. Agonist availability is a function of the concentration of the cells and the rate at which they deplete the medium of agonist. By analogy, that pattern was demonstrated with CSF-1, where the dose response curve for induction of uPA and cell growth is a function of cell concentration [49]. In simple terms, late response genes require more agonist because the cells must receive a continuous supply for the duration of the biological response. When macrophages respond to local LPS exposure *in vivo*, where the agonist is rapidly cleared or diluted by body fluids, or they have engulfed a single microorganism, they give a pulse of cytokines and chemokines that activates the acute phase and causes local inflammation. Conversely, continued exposure of the cells to the stimulus is indicative of the failure of initial defence, and the need to maximally activate the host, including the acquired immune system. When LPS is not cleared, the macrophages progressively induce more and more of the cytokine genes in a manner that is a linear function of the duration of exposure.

The additive nature of signalling pathways in macrophages

As discussed in the introduction, each of the three broad classes of agonist studied here apparently requires the

adaptor protein MyD88 for maximal activity and acts to induce NF- κ B. Despite the shared pathways, maximal activation of the TLR4 pathway, with either CD4-TLR4 or LPS, did not prevent an additive response to the TLR2/6 agonists lipopeptide and peptidoglycan. Conversely, maximal activation of the TLR2/6 pathway with lipopeptide did not block an additive response to LPS. Bacterial DNA effects were additive with either of these classes of agonist. There are relatively few reports on the effects of the combined addition of microbial agonists. In recent studies, two groups studying early gene responses have reported "cross-tolerance" of TLR2 and TLR4-mediated responses [25,26]. In other studies, responses were at least additive. For example, bacterial DNA and LPS were reported to exert more than additive effects on the iNOS gene in macrophages [50]. We have now shown that the effects on cytokines such as TNF- α , IL-6 and IL-12 are also more than additive (Fig 6).

There may be good reason for the innate immune system to retain the ability to respond maximally to different classes of infectious challenge. The global responses to TLR4 and TLR2/6 activation may not be identical (e.g. gram-positive organisms may not induce interferon- β [51]), and the innate immune system may quite commonly respond to multiple classes of challenge in a mixed infection. For example, TLR4-deficient mice display reduced pathology when challenged with mixed infections [52].

From a signaling perspective, the simplest explanation is that the response to any agonist is limited solely or primarily by the availability of surface receptor, and all the downstream effectors are available in excess. Based upon the ability of LPS to repress TLR4 mRNA expression [5], CD4-TLR4 and CD4-TLR2/6 probably act to repress surface expression of the corresponding full-length receptors, thereby preventing super-activation. An alternative is that each pathway associates with a separate functional pool of signalling molecules that need not be identical. If this were the case, one might expect that overexpression of CD4-TLR4 would result in the utilization of more downstream signaling molecules than full activation of endogenous TLR4 could assemble. This appears not to be so since maximal activation by LPS and CD4-TLR4 were remarkably similar. The fact that bacterial DNA action is additive with either CD4-chimaera, or with LPS or lipopeptide, implies that recognition does not absolutely require either TLR4 or TLR2/6. In keeping with this view, macrophages and B cells from knockout mice deficient in either TLR2 or TLR4 respond normally to bacterial DNA, whereas the recent knockout of TLR9 is selectively defective in DNA signalling [10].

Conclusion

We have shown that macrophages exposed to LPS or other microbial agonists do not become refractory to stimulation. Rather, they have entered a new steady state which requires continued stimulation and in which other agonists can generate a further amplification of the response. It may be a characteristic of the "activated" steady state that genes activated during the transition phase from the "inactive" state cannot be reactivated. We have presented an analogy with growth factor responses, where macrophage cells do not respond to CSF-1 with reactivation of the Ets/AP1 pathway unless they have been starved of ligand and maximal cell surface receptor has re-appeared. This is not referred to as CSF-1 tolerance, and the phenomenon that has been studied as LPS tolerance *in vitro* is, based upon our findings, equivalent. This state of tolerance is, in reality, a state of sustained and continued activation.

Materials and Methods

Promoter constructs

The human interleukin 1B promoter clones into the luciferase vector pGL3 was a gift from Dr. Matt Fenton [53]. We subcloned the promoter fragment into the corresponding sites of the Promega renilla luciferase vector. The IL-6 luciferase reporter (pIL6-luc651) was provided by Dr. Oliver Eickelberg [54]. The IL-12 reporter was constructed by us. The murine IL-12 p40 promoter region from -349 to +56 [55] was generated by PCR and cloned into the pGL2-basic KpnI/XhoI site. The ELAM luciferase reporter is as previously described [33].

Transfection

5×10^6 RAW264 cells were transfected by electroporation as described previously [56]. To avoid phenotypic drift in cell culture [23], all experiments were carried out using RAW264 cells that had been recently obtained from the American Type Culture collection, expanded immediately, and then frozen in aliquots. Cells were maintained in culture for no more than 4–6 weeks. All cell culture, including the electroporation procedure, was carried out in RPMI1640 medium with 10 mM HEPES (pH7.4) and 10% fetal bovine serum. The inclusion of HEPES is a departure from previous studies and appears to increase survival and transfection efficiency. In each transfection, we added 10 μ g of the desired firefly luciferase reporter gene, 10 μ g of the IL-1 β promoter-renilla luciferase plasmid and 2 μ g of the selective marker plasmid pNeoTak which also directs expression of the Tet repressor [34]. In this system, only the Neo resistance is relevant, but the Tet repressor could be used subsequently for studies using inducible modifier cassettes. After transfection, the cells were placed in a 100 mm square bacteriological petri dish (Sterilin) with approximately 25 ml of medium. Following overnight incuba-

tion, most viable cells adhere weakly. The medium was changed, and G418 (200 µg/ml) was added. The cells were left in culture with one change of medium to remove dying cells around day 4–5. By day 7–10, several hundred individual foci of stably transfected cells were evident in each dish. These were removed by washing of the surface using a syringe with an 18 g needle (the cells are weakly adherent to bacteriological plastic), expanded and frozen in aliquots for future experiments.

For studies of the dominant-positive TLR receptors, RAW264 cells were transiently transfected by electroporation using 2 µg of the desired expression plasmid encoding the CD4-TLR chimaeric receptor described in Ozinsky *et al.* [35] together with 10 µg of the IL12 promoter luciferase vector. Cells from one transfection were plated into 1 ml of medium in 24 wells for 4–6 hours, the medium was changed to remove dead cells, and the cells were then incubated overnight. The desired agonist was added to the cells without further change of medium, and the cells were harvested 8 hrs later for luciferase determination.

Luciferase assays

For analyses of the response to various agonists, 2×10^5 of the transfected RAW264 cells were placed in 1 ml of medium in 24 well tissue culture plates and incubated overnight. In some experiments, the medium was replaced with prewarmed medium. Agonist at the desired final concentration was added in a small volume, and the cells were incubated for the desired time at 37°C. Cells were harvested and analysed simultaneously for firefly and renilla luciferase activity using the Promega Dual Luciferase reporter assay reagents. In each experiment, duplicate wells were tested; results generally differed by less than 10% of the mean.

Analysis of cytokine production by RAW264 cells and primary macrophages

Bone marrow-derived macrophages (BMM) were derived from the femurs of adult BALB/c mice (Harlan Olac, Bicester, UK) by plating bone marrow cells in fresh medium containing 10% L929 medium as a source of CSF-1 on 10 cm bacteriological plastic plates (Bibby Sterilin, Staffordshire, UK) for 7 days in a 37°C incubator containing 5% CO₂. LPS from *Salmonella minnesota* (Sigma, Poole, UK) was used at a final concentration of 100 ng/ml in all cell culture experiments and *E. coli* DNA (Sigma) was used at a final concentration of 5 µg/ml in cell culture. For control experiments, *E. coli* DNA was digested to completion at 37°C with DNase I (Roche, Indianapolis, IN) in 10 mM Tris-Cl, 10 mM MgCl₂ and 1 mM DTT. Phosphodiester oligodeoxynucleotides (Sigma-Genosys, Poole, UK) were used at a final concentration of 3 µM in cell culture. Oligodeoxynucleotides used were

activating oligonucleotide-1 (AO-1; 5'-GCT CAT GAC GTT CCT GAT GCT G-3') and nonactivating oligonucleotide (NAO-1; 5'-GCT CAT GAG CTT CCT GAT GCT G-3', [9]). RAW264 cells were plated out at 2×10^5 cells per well and BMM at 5×10^5 cells per well in 1 ml complete medium (in the case of BMM, medium contained 10% L929 medium) in 24 well plates on the evening before the experiment. The following day cells were stimulated for 24 h as outlined in the figure legends, supernatants were collected and IL-6, IL-12 and TNF-α levels were determined by ELISA using paired antibodies (Pharmin-gen, San Diego, CA).

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